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Environmental Research 180 (2020) 108676

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

The effect of talc particles on phagocytes in co-culture with ovarian cancer cells

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ARTICLE INFO

Keywords:
"Talc"
"Titanium dioxide"
"Concentrated urban air particulates"
"Diesel exhaust particles"
"Ovarian cancer"
"Macrophages"
"Phagocytes"
"Immunosurveillance"
"Tumoricidal"

ABSTRACT

Talc and titanium dioxide are naturally occurring water-insoluble mined products usually available in the form of particulate matter. This study was prompted by epidemiological observations suggesting that perineal use of talc powder is associated with increased risk of ovarian cancer, particularly in a milieu with higher estrogen. We aimed to test the effects of talc vs. control particles on the ability of prototypical macrophage cell lines to curb the growth of ovarian cancer cells in culture in the presence of estrogen.

We found that murine ovarian surface epithelial cells (MOSEC), a prototype of certain forms of ovarian cancer, were present in larger numbers after co-culture with macrophages treated to a combination of talc and estradiol than to either agent alone or vehicle. Control particles (titanium dioxide, concentrated urban air particulates or diesel exhaust particles) did not have this effect. Co-exposure of macrophages to talc and estradiol has led to increased production of reactive oxygen species and changes in expression of macrophage genes pertinent in cancer development and immunosurveillance. These ffndings suggest that in vitro exposure to talc, particularly in a high-estrogen environment, may compromise immunosurveillance functions of macrophages and prompt further studies to elucidate this mechanism.

1. Introduction

Macrophages (MΦ) phagocytize foreign particles and destroy malignant cells (Dunn et al., 2004); however, it is not often that these two activities are analyzed in the same context. This study was prompted by the epidemiological observation that cosmetic talc powder may be contributing to the risk of ovarian cancer (OC) (Penninkilampi and Eslick, 2018): we tested the hypothesis that interaction with talc particles compromises the MΦs by reducing their anti-tumor abilities.

Talc (hydrous magnesium silicate) is a mined substance considered 'inert' and used in cosmetic products including baby powder. Until 1970's talcum powder may have been contaminated with asbestos, which prompted the International Agency for Research on Cancer (IARC) to declare it carcinogenic to humans (class 1). Since approximately this time talc has been thought to be asbestos-free; nevertheless the IARC concluded that even talc not containing asbestos is possibly carcinogenic to humans (class 2b) (Baan et al., 2006), however the mechanisms were not entirely clear. Dozens of epidemiologic studies (Booth et al., 1989; Chang and Risch, 1997; Chen et al., 1992; Cook et al., 1997; Cramer et al., 1999; Godard et al., 1998; Harlow et al., 1992; Harlow and Weiss, 1989; Mills et al., 2004; Ness et al., 2000a; Purdie et al., 1995; Rosenblatt et al., 1998; Tzonou et al., 1993; Whittemore et al., 1988; Wong et al., 1999; Gertig et al., 2000; Hankinson et al., 1993) have identiffed a 35% increase in ovarian cancer (OC) risk for women who used cosmetic talc powder in the genital area (Cramer et al., 2016; Langseth et al., 2008). While the association is being actively debated (Muscat and Huncharek, 2008), a recent epidemiologic study suggests the association is stronger for women who were premenopausal or were postmenopausal but taking estrogen replacement therapy (Cramer et al., 2016). It is estimated that

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https://doi.org/10.1016/j.envres.2019.108676
Received 6 July 2019; Received in revised form 15 July 2019; Accepted 15 August 2019
Available online 22 August 2019
0013-9351/

genital use of talc might account for 10–11% of OC cases in this country each year (Cramer et al., 1999); OC has a signiffcant contribution to the quality of life and surgical burden of disease.

The mechanism behind this link is unknown; but there are some insights. First, there is clear evidence that particles the size of talc, if they are present in the vagina, easily traverse to the upper female genital tract (Henderson et al., 1971; Heller et al., 1996; Edelstam et al., 1997; Sjosten et al., 2004; Cramer et al., 2007; McDonald et al., 2019a, 2019b). Second, the talc association was more apparent in pre-menopausal women and those postmenopausal women who were taking estrogen replacement therapy (Cramer et al., 2016) suggesting higher estrogen may ignite the pathogenesis (Berge et al., 2018). Third, some but not all experimental data suggest that talc particles are not completely innocuous: (NTP, 1993; Hamilton et al., 1984; Frazier-Jessen et al., 1996).

The epidemiologic data are at odds with the perception of talcum powder as a relatively inert, insoluble cosmetic substance that appears to have been well-tested and safe - as a chemical. However it is possible that talc, while it may not be directly mutagenic as a chemical compound (Boorman and Seely, 1995; Pickrell et al., 1989), is a hazardous factor as a particle. The approach and assays we used here stem from our prior interest in particulate matter and how macrophages interact with particles (e.g. (Zhang et al., 2015; Fedulov et al., 2008)); here we focused not on the process of carcinogenesis but rather on the immunotoxic effect of talc. Our hypothesis is that, in a high-estrogen environment, exposure to talc particles alters MΦ functions to permit increased survival of malignant cells. We postulate this could occur via a release of tissue-damaging factors (e.g. reactive oxygen species, ROX) and/or by compromising immunosurveillance abilities of the MΦs and their tumoricidal effectiveness.

2. Materials and methods

We used phagocytic murine cells lines J774 and IC21 and in some experiments RAW264.7 (ATCC; Manassas, VA) as phagocytes. These lines have been historically used to test the effects of female hormones on MΦs with success (Benten et al., 2001a; Pisetsky and Spencer, 2011; Hayashi et al., 1998). The J774 cells are 'chromosomally female' and thus are a better 'prototypical macrophage' for testing of estrogen effects. The IC-21 cell line was obtained by transformation of normal C57BL/6 mouse peritoneal macrophages (Mauel and Defendi, 1971). This line shares many properties with normal mouse MΦ and displays MΦ-speciffc antigens. IC-21 cells have phagocytic and cytolytic properties, can lyse tumor targets in-vitro (Crawford et al., 1990) and appear to be a terminally differentiated macrophage line (Walker and Demus, 1975; Walker and Gandour, 1980). Hence they are more relevant to OC, however they are 'genetically male' and thus may be less responsive to estrogen although they also express estrogen receptors and respond to estrogen stimulation (Benten et al., 2001b).

These cells were maintained in 100-mm Petri dishes in DMEM (for J774) or RPMI-1640 (for IC21) free of phenol red, supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and 10 mM HEPES.

Tumoricidal eff ciency of the MΦ was tested in a standard MΦ-tumor co-culture using the murine ovarian surface epithelial cell line (MOSEC) ID8 (Roby et al., 2000) provided by Dr. Katherine Roby (University of Kansas). ID8 cells most closely resemble human epithelial form of OC, which contributes to 90% of the cases (Roby et al., 2000; Greenaway et al., 2008). We have transduced these cells with an EF1α-GFP lentiviral construct (GenTarget, Inc.) containing Blasticidin-S deaminase and validated that ffuorescence was at acceptably stable level in preliminary studies (Fig. 5). These cells were maintained in DMEM with stable L-Glutamine (10-101-CV, Corning) and supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), Blasticidin S (10 μg/mL, Gibco) and 'ITS media supplement' containing 1.0 mg/mL recombinant human insulin, 0.55 mg/mL human transferrin (substantially iron-free), and 0.5 μg/mL sodium selenite (1:100) from Sigma-Aldrich.

Talc ($Mg_3Si_4O_{10}(OH)_2$, CAS Registry Number: 14807-96-6, USP grade, particle diameter < 10 μm, was obtained via JT Baker (Batch No: 0000184513) and is certiffed as asbestos-free. The particles were suspended in PBS and ffltered through 30 μm nylon mesh fflters (no visible loss of material has occurred). We did not use any commercial talc products.

Titanium dioxide (TiO_2), CAS Registry Number 13463-67-7, control particles (with mean particle size of ~1 μm) were a gift from Dr. L. Kobzik (Harvard School of Public Health, Boston, MA); these were used previously in our studies (Zhang et al., 2015; Fedulov et al., 2008).

Concentrated urban air particles (CAP) were obtained via Harvard School of Public Health particle concentrator (batch #816) and represent urban contaminants typically present in Boston air (Zhou and Kobzik, 2007; Imrich et al., 2000; Sigaud et al., 2007). They were suspended in PBS and used as is without ffltering or sterilization.

Diesel exhaust particles (DEP) were generously provided by Dr. Ian Gilmour at the U.S. Environmental Protection Agency and used by us in earlier studies (Fedulov et al., 2008; Gregory et al., 2017). They were also suspended in PBS and used as is without ffltering or sterilization.

All particles were of comparable "ffne" size although not identical, see Fig. 1. All particles were sonicated on ice to break up clumps using Qsonica Q55 probe sonicator.

Prior to experiments the cells were serum-starved for 24 h in Macrophage-SFM (serum free medium) (Gibco/Life Sciences). Adherent cells (in black-walled 96-well tissue culture treated plates, Corning) were then treated to 17-β estradiol (E2) (cell culture grade, Sigma Aldrich) in a range of concentrations from 10 to 0.0001 μg/mL; ethanol served as vehicle control. Talc (or control particle) suspension was added at the same time as estradiol in doses from 0.1 to 20 μg/well in dose-response experiments and in dose 10 μg/well otherwise. Detection of reactive oxygen species (ROX) was performed after 4 h via Cell ROX Green Flow Cytometry Assay (Molecular Probes). Viability analysis and cell count veriffcation were done after 24 h of incubation via staining with Annexin V and Sytox (Invitrogen). RNA isolation (via RNEasy kit, Qiagen) for gene expression testing was done after 24 h as well.

Co-cultures with MOSEC-GFP cells continued for 72 h; MOSEC-GFP cells were added at 5:1 (MΦ:MOSEC) ratio; particles were almost entirely phagocytized by MΦs by this time (Fig. 1 Panel 2) therefore we do not assume that MOSEC cells were exposed to particles. Medium with fresh estradiol (at the same concentration as the original) was replaced every 24 h to compensate for the estradiol decay. At 72 h the cells were detached (TrypLE, Lonza), washed once with phenol red – free RPMI containing 10% FBS and resuspended in ffow cytometry (FACS) buffer (PBS + 0.5% bovine serum albumin, Gibco) for analysis.

Flow cytometry was performed using MACSQuant Analyzer cytometer (Miltenyi) running MACSQuantify Software V2.11. Samples were gated based on their forward and side scatter to exclude the smallest debris and large clumps. The analysis region (gate) distinguished GFP-bright MOSEC cells from mildly autoffuorescent MΦ; we calculated percentage and mean ffuorescence intensity (MFI) in the GFP channel for the GFP-bright MOSEC region. The integral ffuorescence index was calculated as a product of 'percent positive' multiplied by 'MFI value' and reffects the ratio of surviving GFP-MOSEC cells normalized to the number of MΦs in combination with the extent of GFP transgene expression (Csepregi et al., 2018; Kamau et al., 2001). Talc particles did not contribute to the ffuorescence signal (Fig. 5).

Microscopy. To visualize engulfment of talc particles the cells were treated with talc suspensions as described. After 24 h the cells were detached by trypsinization, and centrifuged onto standard microscopy slides (VWR) via Cytospin II (Shandon). The slides were ffxed with methanol and stained by Diff-Quik, a version of Romanowski stain. The images were made on an Olympus BH-2 light microscope with attachments for polarized light microscopy and an Olympus Q-Color 5 camera. All pictures were taken with the same degree of partially

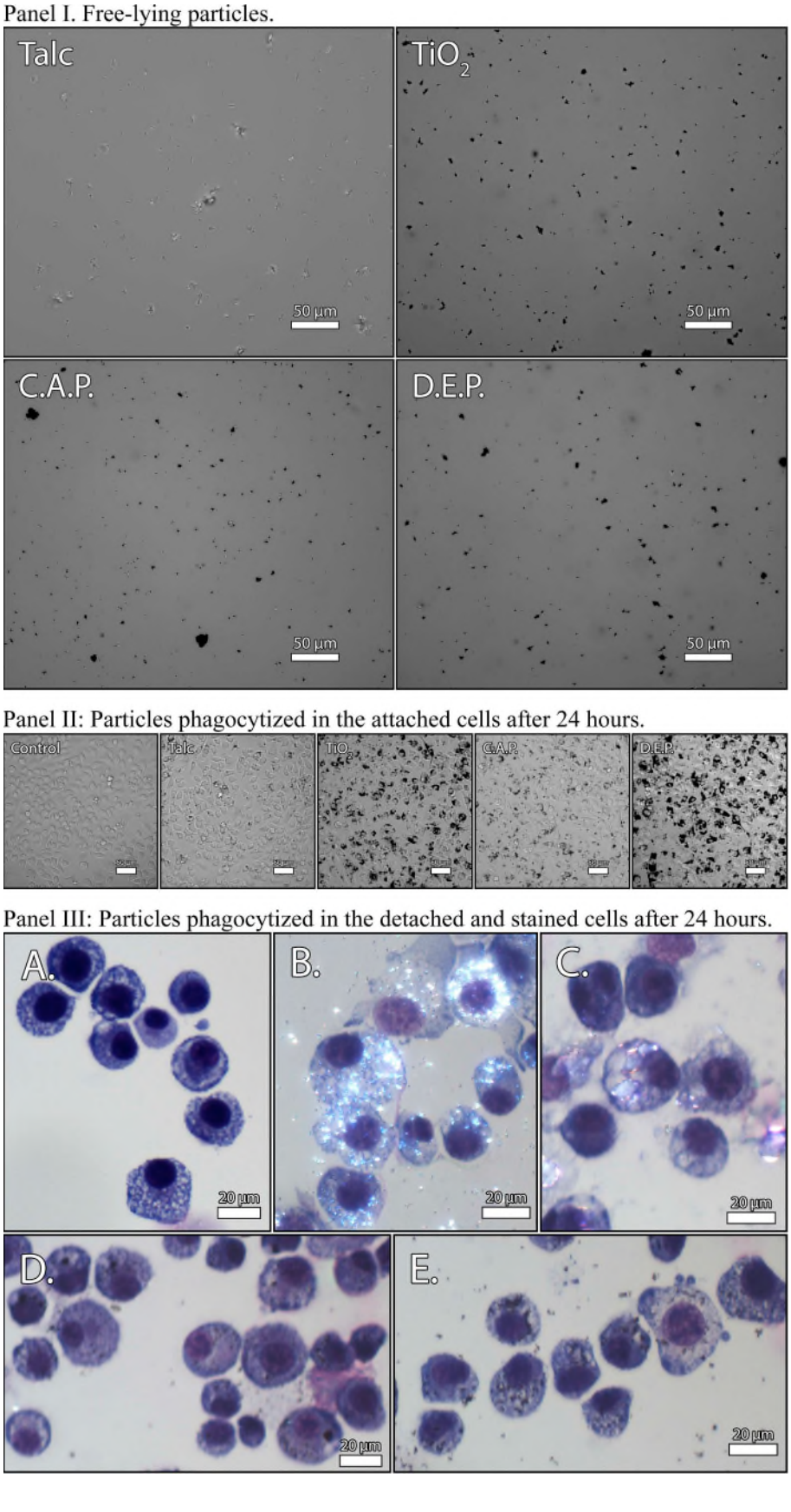

Fig. 1. Microscopic observation of particle phagocytosis.
Panel I: Free-lying particles. Particle suspensions were sonicated and plated freely in 300 µL of PBS, allowed to settle to the bottom for 1 hr and photographed using Nikon Eclipse Ti2 microscope. 400X.
Panel II: Particles phagocytized in the attached cells after 24 hours. Attached cells were treated to particle suspensions and allowed 24 hours to phagocytize. Nikon Eclipse Ti2; 400X.
Panel III: Particles phagocytized in the detached and stained cells after 24 hours. J774 cells without particles (A) and after 24 exposure to TiO_2 (B), talc (C), CAP (D) and DEP (E). Cytospin slides. Staining: Diff-Quik, original magniffcation for all images: 400X, on all images-bar: 20 µm. Olympus BH-2 light microscope with attachments for polarized light microscopy and an Olympus Q-Color 5 camera. All pictures were taken with the same degree of partially crossed polarizers so that black particles, birefringence of particles, and the cells could be seen. Note that talc and TiO_2 particles are birefringent, and single birefringent particles were seen in CAP and DEP preparations.

crossed polarizers so that black particles, birefringence of particles, and the cells could be observed.

To visualize the co-cultures with ffuorescent MOSEC cells with and without talc and control particles we used Nikon Eclipse Ti2 microscope with associated camera and software.

Gene expression proffling was achieved via Cancer PathwayFinder

RT2 Proffler PCR Array (Qiagen) which interrogates 84 cancer-pertinent genes using the CFX96 real-time PCR system (Bio-Rad) and CFX Manager 2.0 software (Bio-Rad). Raw Cq values for all genes (GOI) were normalized to an average of 5 housekeeping genes (HKG): Actb, B2m, Gapdh, Gusb and HSP90ab1 (NormΔCq = (Cq(GOI) - Ave Cq (HKG)). Expression values were obtained using formula 2^(-NormΔCq)*1000. These values were assembled into a matrix to become an input ffle for statistical analysis via TIGR Mev 4.9 (Saeed et al., 2003). Data were analyzed via Pavlidis Template Matching (PTM) method (Pavlidis and Noble, 2001) using the threshold p-value 0.05. In the heatmaps, red color indicates higher expression, green – low expression; row-normalized color intensity is proportional to the value for each gene in each sample.

All co-culture experiments were repeated more than 3 times. Each measurement was done in duplicate or triplicate. Triplicate RNA samples were selected from one representative experiment for gene expression analysis. Data are presented as Mean ± SEM. Data plotting and statistical analysis (other than array data) was performed using Excel 2007 (Microsoft) and Prism 7.02 (GraphPad Software); statistical signiffcance was accepted when $p < 0.05$. To estimate signiffcance of differences between groups we used the non-parametric Mann-Whitney *U* test, one-way or two-way ANOVA with Tukey, Fisher or HolmeSidak tests, or Kruskal-Wallis ANOVA with Dunn's or Dunnett's test as dictated by the number of groups, data normality and experimental question.

3. Results

3.1. Effect of talc and estradiol on the phagocytes

MΦs were treated with vehicle alone (ethanol), talc alone, estradiol (E2) alone, or the combination of E2 and talc (Fig. 1). Costimulation of MΦs with estradiol (E2) and talc produced an additive effect on ROX production (Fig. 2). While control TiO_2 particles were also phagocytized, the production of ROX was only slightly increased in J774 cells and not increased in IC21 cells (not tested in RAW264.7).

Gene expression proffling was performed via PCR-array aimed at detection of genes relevant in cancer pathways (see Qiagen PAMM-033ZD for the full list). Fig. 3B demonstrates a cluster of genes signiffcantly upregulated by talc in the two types of phagocytes: interestingly in J774 cells the effect of talc was prominent with or without E2, when in IC21 cells the co-effect of talc and E2 is better seen. When examining both cell types, we found patterns of similarity in the increased expression of this set of genes. Quite notably this cluster involves genes of extracellular, outer-membrane and releasable nature that are pertinent in carcinogenesis (see Discussion for details).

Fig. 3A demonstrates a cluster of genes co-inhibited by talc and E2, suggesting a strong co-effect of particles and the hormone, but also (more so for J774 cells) the effect of talc alone. Many of these genes encode intracellular factors pertinent to immunosurveillance, see Discussion for details. Many of the genes (but not all) were affected similarly in all three or in two out of the three cell types we tested.

In summary, talc alone and especially in combination with E2 produced changes in gene expression that may promote pro-tumorigenic environment and less eff cient surveillance (tumoricidal) activity of the macrophages.

Exposure of MΦs to talc or E2 did not lead to signiffcant increases in staining with Annexin V or Sytox (Fig. 4) or any noticeable changes in cell numbers in the 24 h period; the exceptionally high doses did occasionally decrease the viability of the MΦs (however slightly), hence we did not employ these concentrations in further experiments. Some variability in this staining is reported in Fig. S2.

3.2. Effect of phagocytes pre-treated with talc and estradiol on MOSEC ID8 cells

Wildtype MOSEC ID8 cells were transduced to express GFP under EF1a promoter. GFP^+ MOSEC ID8 cells were added for 72 h with addition of fresh E2 every 24 h of that period. Visualization of the co-culture was performed via an Eclipse Ti2 UV microscope (Nikon) with associated camera and software (Fig. 5). Detection of surviving GFP^+ MOSEC cells was performed via ffow cytometry.

MΦs were treated with particles in the presence of estradiol (E2) or vehicle, as before; in control samples talc was replaced by TiO_2, CAP or DEP particles.

In dose-response experiments we observed that talc and E2 have potentiated the effect, the magnitude varied depending on the MΦ cell line but the ffndings (Fig. 6) reffect that both substances had dose-response kinetics. IC21 cells did not appear as sensitive to E2 as J774.

Fig. 7 demonstrates that neither particle had a statistically signiffcant cytotoxic effect at 10 μg/well with or without E2 at 24 h (immediately before MOSEC cells were added). We report a microscopic observation that talc-treated cells appeared more fragile than any controls. Microscopically and via ffow cytometry we also report that most particles were phagocytized at this timepoint, with only single particulates remaining outside the cells. MOSEC-GFP cells were then added and co-cultured for 72 h.

Our key ffnding is presented in Fig. 8. A combination of talc and E2 (but not of control particles and E2) has allowed signiffcantly increased MOSEC-GFP readings compared to especially the vehicle-only control where most MOSEC cells were eliminated from the co-culture. Of note, talc alone tended to be effective (albeit not statistically signiffcant in all experiments or pooled data), especially for IC-21 cells. The particles alone (when no MΦs were present) did not signiffcantly affect the numbers of MOSEC cells after 72 h; there was a trend towards a slight decrease in cell numbers (Fig. 7G).

In a subset of experiments (with IC21 cells) we recorded the number

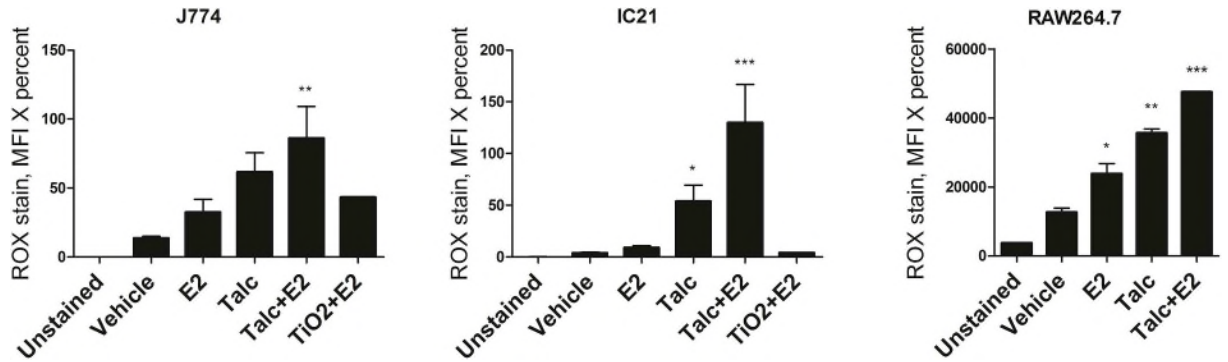

Fig. 2. Production of ROX at 4 h (ffow cytometry) was enhanced by either E2 or talc alone, the effect was additive. n = 2/group. *P < 0.05, **P < 0.01, ***P < 0.05 (Tukey).

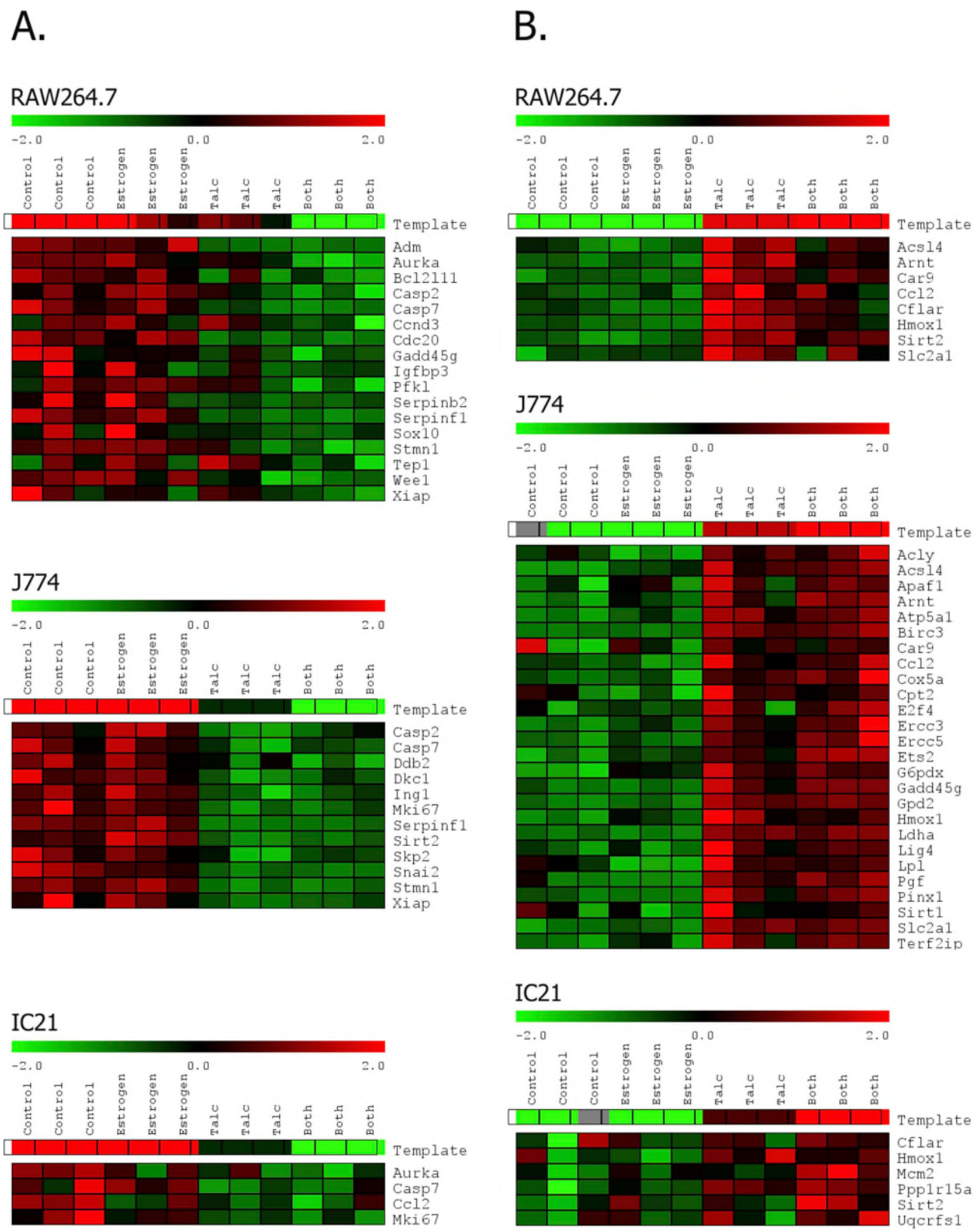

Fig. 3. PCR array profiling at 24 h exposure. Gene expression values were analyzed using Pavlidis Template Matching (PTM) with a threshold p-value of 0.05. Color is proportionate to gene expression (green = lowest, red = highest). A. Inhibitory effect: We aimed to identify genes most inhibited by the combination of estrogen and talc, but also affected by talc particles alone: the expression pattern of Aurka served as the template for RAW264.7 cell samples; matching template values (control: 0.9, estrogen: 0.9, talc: 0.4, both: 0) were used for J774 and IC21 cell samples. B. Stimulatory effect: Similarly, the template (control: 0, estrogen: 0, talc: 0.8, both: 1) aimed to identify targets most upregulated by the combination of estrogen and talc, but as well those increased by talc particles alone. Each sample tested is shown individually: N = 3 per group, total N = 36.

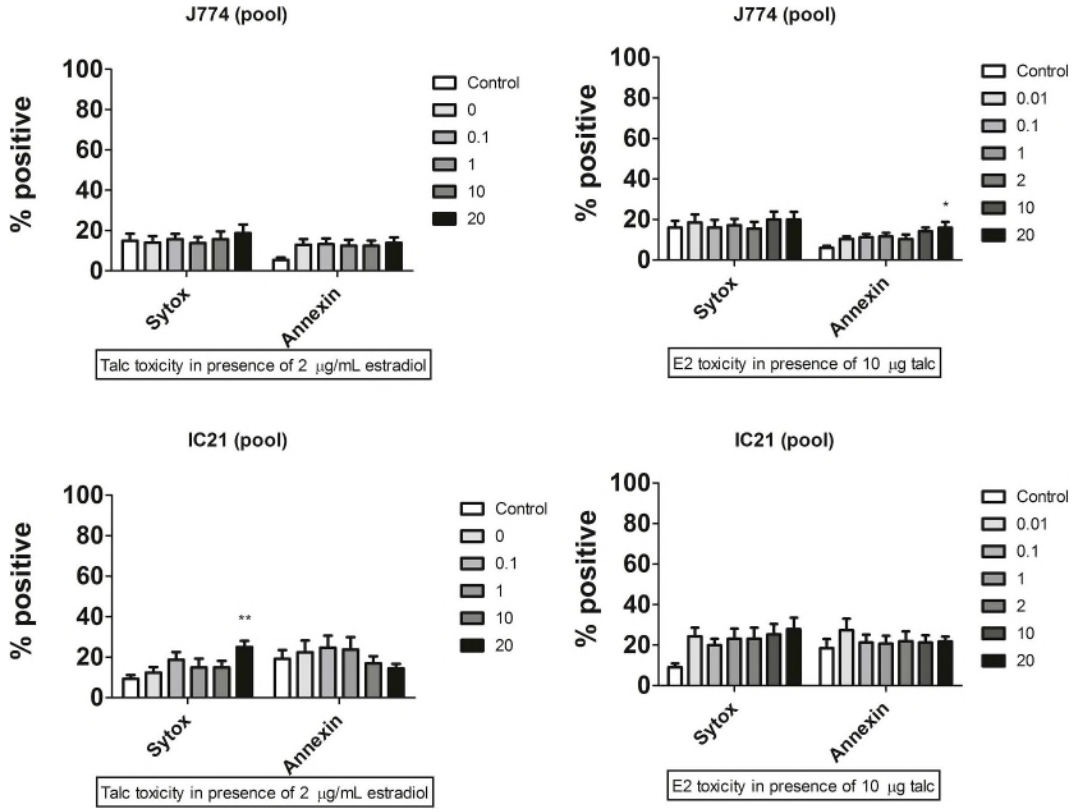

Fig. 4. Dose-response cytotoxicity analysis. Talc and E2 were not signiffcantly toxic to macrophages alone or in combinations used. J774 cells or IC21 cells were exposed for 24 h to either increasing doses of estradiol in presence of 10 μg/well of talc, or to increasing doses of talc in presence of 2 μg/mL of estradiol. Cells were stained for apoptosis and necrosis via Annexin V and Sytox assay kit; ffow cytometry determined the percentage of positive cells plotted here. Pooled data from three experiments are shown. n = 6 to 8 per group. *P < 0.05; **P < 0.01 (Dunn).

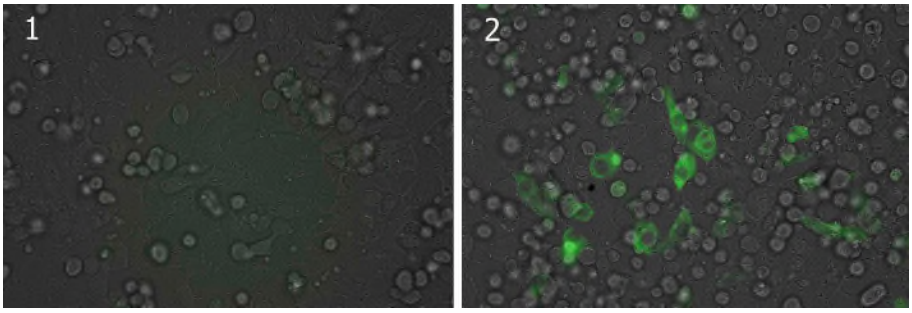

Fig. 5. Co-culture visualization: co-culture of IC21 macrophages with wildtype (1) or GFP$^+$ MOSEC cells (2) with talc photographed at the same setting in the FITC/GFP channel. Magniffcation X400; Nikon Eclipse Ti2.

of GFP$^+$ MOSEC (per microliter of cytometry buffer) and it was consistently (with ffuorescence) higher in the wells where MΦs were treated with Talc + E2 but not with TiO_2 + E2 or E2 alone (Supplementary Fig. 1).

In a validation experiment we used an alternative approach which did not involve a GFP transgene. RAW 264.7 MΦs were treated to talc particles and E2 similarly and co-cultured with wildtype MOSEC cells. After a 72-h co-culture the MOSECs were labeled with Calcein AM whereas the MΦs were labeled with anti- Ly6-C and anti-CD45; a proliferation ratio calculation revealed that the combination of talc and E2 allowed a larger proportion of MOSEC cells than either agent alone (Fig. S3).

In summary, a combination of talc and E2 especially, and in some cases talc alone, affected the MΦs to permit higher MOSEC-GFP survival.

C, D: Cytotoxicity analysis. IC21 or J774 cells were treated to particles alone or in combination with E2 for 24 h and analyzed via Sytox Green and Annexin V PE staining. N = 3 per group.

4. Discussion

This is the ffrst study linking the macrophage, talc particles and estrogen in a potential mechanism to explain the effect of talc behind the ovarian cancer statistics seen in epidemiology studies. Histology of surgically resected tissues shows that in the setting of known exposure, talc has been capable of migrating from the perineum to pelvic lymph nodes, ovary, fallopian tube, uterus and cervix (Cramer et al., 2007; McDonald et al., 2019a, 2019b); however carcinogenicity studies indicated that prolonged exposure to talc inhalation by some experimental animals does not induce cancer (Hamilton et al., 1984; Frazier-Jessen et al., 1996; Boorman and Seely, 1995; Pickrell et al., 1989) although some tumors, tumor-like morphological changes and

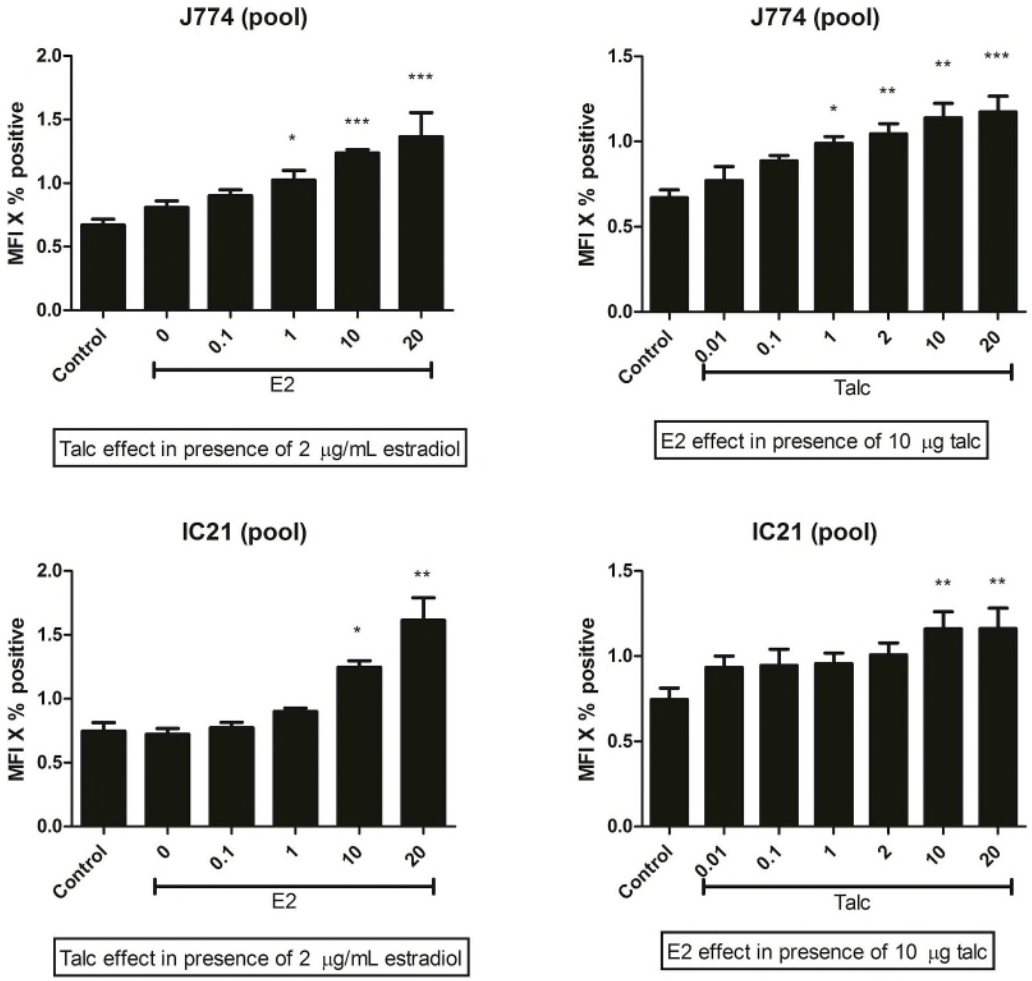

Fig. 6. Dose-response efiect in the co-culture. J774 cells or IC21 cells were exposed for 24 h to either increasing doses of estradiol in presence of 10 μg/well of talc, or to increasing doses of talc in presence of 2 μg/mL of estradiol. After preincubation, ffuorescent MOSEC ID8 GFP^{+} cells were added for 72 h; medium and estradiol were replaced every 24 h. Flow cytometry at the end of co-culture recorded the percentage of GFP-positive cells and their MFI; the plots represent the product (MFI X percent). Data values from four experiments were normalized to average and pooled, n = 4/group. *P < 0.05; **P < 0.01; ***P < 0.005 (Dunn).

macrophage activation were reported (Shim et al., 2015; NTP, 1993; Hamilton et al., 1984).

Three particular lines of evidence argue that the estrogen milieu may determine the effects of talc: A) in humans, the talc association was more apparent in premenopausal women and those postmenopausal women who were taking estrogen replacement therapy (Cramer et al., 2016); B) in rodents, lung tumors developed in female, not male rats exposed to talc (NTP, 1993); and C) our own work indicating that estradiol (E2) affects MΦ uptake of particles (Zhang et al., 2015). Notably, there is no literature to suggest asbestos-free talcum powder causes any cancers in men.

Here we focused on the MΦ because A) MΦs are the ffrst to encounter and engulf talc particles; once phagocytized, these particles persist inside the MΦ (Goldner and Adams, 1977); B) MΦs are part of innate immunity responsible for the removal of aberrant, malignant cells (Dunn et al., 2004); they are especially active when primed (Hagemann et al., 2008). C) MΦs produce aggressive molecules capable of driving persistent tissue damage; and D) in patients with ovarian tumors, talc is observed within MΦs (Cramer et al., 2007).

Of note, the literature does not suggest an association of chronic pelvic inffammatory diseases with perineal talc use (Merritt et al., 2008), indicating that typical cytokine pathways are unlikley to make a signiffcant contribution (although inffammation can be a contributing factor in OC (Ness et al., 2000b)). Moreover, in a typical model, the MΦs are co-cultured over a large amount of tumor cells which leads to alternative activation (M2) phenotype, also called tumor-associated MΦs (Hagemann et al., 2006). These cells have distinct expression proffles and may be a suitable model to study processes in *established* tumors, whereas we are focused on the *onset* of the process. We emphasize that E2 pre-treatment does not affect this polarization *per se* (Wang et al., 2015); in our preliminary studies markers of M1 vs. M2 phenotype were unchanged (not shown). This is consistent with our hypothesis that combination of talc and E2 produces an effect in MΦs that is distinct from the heavily studied alterations.

Here we hypothesized that in a high-estrogen environment the talc particles alter MΦ function and decrease the killing of OC cells. We postulated this could occur via either a release of damaging factors that promote formation of aberrant (OC) cells, and/or via compromised immunologic surveillance (tumoricidal) ability of the MΦ, which could allow aberrant cells (that regularly appear in low numbers in the organism) to develop into clinical tumors. The latter premise was supported in part by a report that exposure of MΦs to talc can inhibit their phagocytic activity (Beck et al., 1987).

We found that talc and estradiol co-enhanced the production of ROX which participate in cell growth/proliferation, differentiation, protein synthesis, glucose metabolism and survival of malignant cells (Liou and Storz, 2010); ROX play important role in the pathogenesis of OC (Saed et al., 2017). This ffnding is consistent with in vivo data (Shim et al.,

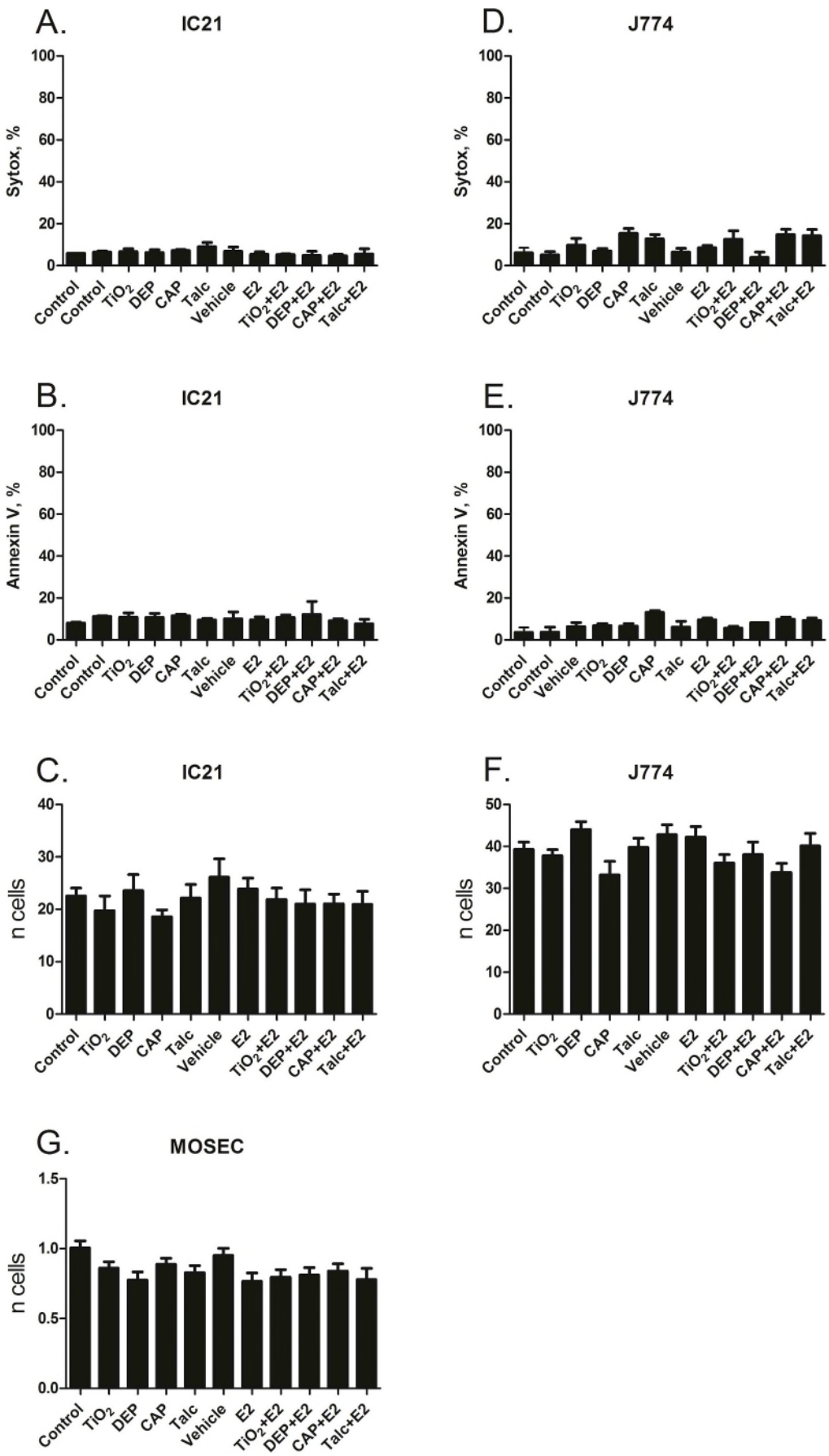

Fig. 7. Cytotoxicity analysis. IC21 cells (A, B) or J774 cells (D, E) were treated to particles alone or in combination with E2 for 24 hrs and analyzed via Sytox Green and Annexin V PE staining. n = 3 per group. Cell number: IC21 (C) or J774 cells (F) were visually counted in a haemocytometer after 24 hr incubation with particles and estrogen. Normalized average of 3 repeat experiments; N = 6 per group for IC21cells, n = 7 per group for J774 cells. G: The effect of particles on cell counts of MOSEC cells treated alone. MOSEC cells were visually counted in a haemocytometer after 72 hr incubation with particles and estrogen. Normalized average of 3 repeat experiments; n = 6 per group.

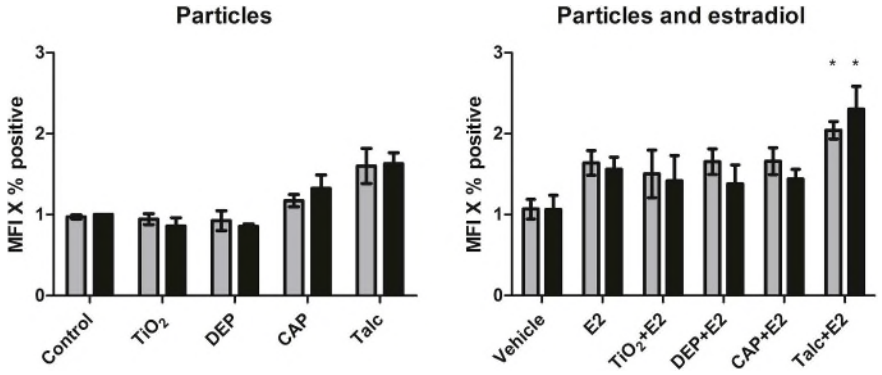

Fig. 8. The effect of talc and control particles in co-culture of MΦ and MOSEC cells. J774 cells or IC21 cells were exposed for 24 h to 2 μg/mL of E2 (or vehicle) and to 10 μg/well of talc, TiO_2, CAP or DEP particles, or combination. After 24-h pre-incubation, ffuorescent MOSEC ID8 GFP^+ cells were added for 72 h; medium and estradiol were replaced every 24 h. Flow cytometry at the end of co-culture recorded the percentage of GFP positive cells and their MFI; the plots represent the product (MFI X percent). Data values from three experiments were normalized to control and pooled, n = 3/group. *P < 0.01 vs. Vehicle (two-way ANOVA with Tukey or Holm-Sidak).

2015).

Moreover, talc alone, and to some extent in concert with estradiol has upregulated a cluster of genes that encode factors of releasable, extracellular or outer-membrane nature whose increase alters the extracellular milieu and contributes to tumor growth and metastasis: 1) Carbonic anhydrase *Car9*: enhances extracellular acidity and promotes tumor growth (Swietach et al., 2007, 2009); 2) HMOX1: macrophageal heme oxygenase-1 in tumor microenvironment can dictate cancer growth and metastasis (Nemeth et al., 2015); 3) Solute carrier family 2 facilitated glucose transporter member 1 (SLC2A1), a membraneous protein which promotes tumor cell proliferation and metastasis (Yan et al., 2015); 4) CFLAR, a gene that encodes Cellular FLICE-inhibitory protein (CFLIP), remarkably associated with carcinogenesis including OC (Lozneanu et al., 2015); 5) Sirtuin 2 (SIRT2) – a known therapeutic target in cancer (Jing and Lin, 2016).

At the same time, and perhaps more importantly, we found that talc and estrogen co-inhibited expression of a cluster of genes responsible for intracellular, internal proteins playing a role in anti-tumor immunosurveillance. The cluster includes 1) *Aurka* - Aurora kinase A, an intracellular protein which regulates proliferation and ability to develop the 'anti-tumor' M1 phenotype by the MΦs (Ding et al., 2015; Sica and Mantovani, 2012). 2) *Gadd45g* - Growth arrest and DNA damage-inducible 45, an intracellular protein involved in MΦ maturation; its deffciency causes less eff cient tumor immunosurveillance (Schmitz, 2013); although the expression change for this gene was cell-type dependent; 3) Casp7 (Caspase-7) – a protein playing role not only in apoptosis, but also important in MΦ phagocytosis: Casp7-deffcient macrophages show impeded completion of phagocytosis (Akhter et al., 2009); 4) CDC20 (Cell division cycle 20) - a regulatory protein shown to be upregulated in MΦ recruited into the tumor and, comparatively, downregulated in those MΦ not engaging with the tumor (Poczobutt et al., 2016); 5) Mki67 – a known proliferation marker; 6) Stmn1 (Stathmin 1) is involved in cell cycle regulation and its inhibition leads to a decrease in proliferation as it is involved in microtubule stability inside the cell (Rubin and Atweh, 2004); Stmn1 affects how MΦs are activated (Xu and Harrison, 2015); interestingly, micro-RNA targeting Stmn1 can be transferred from MΦs to tumor cells (Aucher et al., 2013); 7) XIAP (X-linked inhibitor of apoptosis protein) is important in resistance to cell death in MΦs and is generally involved in MΦ innate immune functions (Rijal et al., 2018).

In combination, our gene expression data indicate both an "outward effect": induction of releasable extracellular deleterious factors, as well as an "internal effect": inhibition of important intracellular factors. Hence, this exploratory proffling has provided us with a hypothesis that together these effects can create preferential conditions for the survival of OC cells in co-culture. Our expression proffling was not comprehensive: a whole transcriptome analysis is needed to uncover full details of the deregulation in the MΦs. We also did not aim to determine whether the changes we found are unique to talc. The focus of our experiments was to demonstrate whether talc is inert when phagocytized in high-estrogen milieu, and we conclude that it is not inert.

In co-culture experiments, we determined that co-exposure of the MΦ to talc and E2 permits higher numbers of OC (MOSEC ID8) cells to survive. We ffrst determined whether E2 and talc had any effect on the MΦ viability in monoculture. Talc or E2 had no toxic effect seen as either apoptosis or necrosis rate, aside of a slight change at 20 μg/mL E2; we have not used this excessive concentration further to assure that the viability of pre-exposed MΦs is the same in all samples. We noted that MΦs, which especially avidly phagocytized talc, had a slight morphological change in appearance, as seen in Fig. 1, which however did not lead to signiffcant changes in counts (Fig. 7).

Hence, we treated the MΦ with a combination of 10 μg talc per well and 2 μg/mL E2, and in subsequent co-culture the ffuorescence of GFP^+ MOSEC ID8 cells and their percentage were higher after 72 h (indicating their better survival) compared to controls where MΦ had been treated with vehicle alone or with either agent alone. When talc was replaced with control particles - TiO_2, CAP or DEP, the effect was also not seen (Fig. 8).

In dose-response experiments, the J774 cells, 'chromosomally female', appeared dose-responsively susceptible to the effect of E2 and talc, whereas the 'chromosomally male' IC21 were mostly susceptible to talc. In both cells, we note, even the lowest dose of E2 (1 ng/mL) has boosted (albeit not signiffcantly) the effect of talc.

Because the survival of MOSEC cells is dependent on the number of macrophages in a well we mostly relied on the ffuorescence parameters in the FITC/GFP channel, which takes into account the ratio of both cells types as well as the 'brightness' of the GFP transgene as a measure of viability (Csepregi et al., 2018; Kamau et al., 2001). However, in a subset of experiments we also physically counted the MOSEC-GFP^+ cells (see example from one experiment in Fig. S1) and used a transgene-independent method (Fig. S3) and these parameters gave consistent results.

We note that a bolus of 2 μg/mL of E2, although realistic, is likely at the higher end of concentration ranges. In normal mice and humans concentration of circulating E2 in serum is in the range of pg/mL to ng/mL (Wood et al., 2007; Zhang et al., 1999). However tissue levels of steroid hormones may exceed plasma by 20-30-fold (Batra, 1976; Akerlund et al., 1981; Straub, 2007) and ovarian tissue concentration of E2 is more than 100-fold higher than in serum (Lindgren et al., 2002). This may be an indication of why talc use is associated with ovarian cancer rather than at other sites. It is also worth noting that *in-vitro* bioavailability of the hormone from a single administration cannot be directly interpolated dose-wise to the sustained tissue exposure of the resident cells *in-vivo*. We also note that in modeling the effects of E2 sometimes even higher doses have been employed to make for a useful short-term model (Drew and Chavis, 2000).

Our report aims to establish the phenomenon of decreased anti-tumor (anti-MOSEC) activity of the phagocytes after talc and E2 combination pre-treatment; it also partly delineates what further studies are needed to elucidate the speciffc pathways involved into the inhibition of macrophageal activity. In our study we did not investigate carcinogenic properties of talc *per se*. Studies of other sources and batches of

talc as well as with other cell types are needed for a more comprehensive evaluation of the effect. Further research is needed to determine whether and to what extent the effect of talc on phagocytes exists in vivo, particularly in humans; these studies were beyond the scope of our project. We did not investigate whether the inhibited tumoricidal activity we discovered could entail an increased likelihood of tumor growth. However, we believe our ffndings can help reconcile the presumed innocuous nature of talc with epidemiological data on talc powder use and OC risk by suggesting that the effect can be mediated by the macrophages.

The ffndings of this study using phagocytic murine cell lines as prototypical macrophages and MOSECs as prototypical ovarian cancer cells suggest that in vitro exposure to talc particles, particularly in a high-estrogen environment, may compromise the macrophageal immunosurveillance functions. Control particles (titanium dioxide, concentrated urban air particulates or diesel exhaust particles) did not have this effect. Exposure of macrophages to talc and especially co-exposure to talc and estradiol has led to increased production of reactive oxygen species and changes in expression of macrophage genes pertinent in cancer development and immunosurveillance.

Acknowledgements

We thank Dr. Jonathan Reichner, Dr. Alfred Ayala and Dr. Chun-Shiang Chung for helpful discussions.

Abbreviations

MOSEC	murine ovarian surface epithelial cells
DEP	diesel exhaust particles
CAP	concentrated urban air particles
TiO_2	Titanium dioxide
E2	17-β estradiol
ROX	reactive oxygen species
MΦ	macrophage

Data statement

Data supporting the ffndings may be obtained for academic purposes from the corresponding author upon a reasonable request through the editorial off ce after disclosure of the conffict of interest.

Funding

The study was supported in part by a pilot project from the Harvard-NIEHS Center [ES-000002] to AVF and by the Particles Research Core of [ES-000002], by Rhode Island Hospital funds to AVF and BWL, by funds from National Institutes of Health NIGMS [P20GM121344] to BWL and by NHLBI T32 [HL134625] to HW. Funding for JJG in his role in this research was provided through John J. Godleski, MD, PLLC.

Declaration of interest

The authors: AM, DJG, CCM, BWL, HW, LMR and AVF have no competing interests. JJG has served as an independent expert and provided expert testimony in talc and other environmentally related litigation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2019.108676.

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